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Predominance of *Leishmania major* and rare occurrence of *Leishmania tropica* with haplotype variability at the center of Iran

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ABSTRACT

Background: *Leishmania major* is a causative agent of zoonotic cutaneous leishmaniasis in the center of Iran, Abarkouh district. Molecular characterization and precise incrimination of *Leishmania* species was carried out to perform controlling measurements and to design treatment programs for zoonotic cutaneous leishmaniasis.

Methods: All smears isolated from ulcers of suspected patients were examined under a light microscope and graded for amastigotes frequency. Extraction of DNA, PCR, RFLP and sequencing of ITS-rDNA genotype were done to increase the efficacy of *Leishmania* parasites identification at their species-specific level and to detect any *Leishmania* infections within.

Results: Humans were found to be infected with *L. major* with high infection frequency and also *Leishmania tropica* was identified with low occurrence for the first time as non-native species using molecular analyses. The rates of infections was considerable with microscopic observation ($n = 65$, 73%) out of 89 smears prepared from suspected patients. Molecular analyses showed that the density of *L. major* was significantly higher ($n = 48$, 53.93%) than *L. tropica* ($n = 4$, 4.49%) (Mann–Whitney *U* test: $p < 0.05$) and two samples (2.25%) remained ambiguous after several sequencing. *L. major* did not have diversity with two common haplotypes but *L. tropica* were found to exhibit high diversity with three novel haplotypes.

Conclusion: *L. major* was considered the causative agent of leishmaniasis in the region, but the identification of a non-native *L. tropica* revealed the importance of further isolation of *Leishmania* parasites following molecular analyses and confirmation, revealed the importance of further isolation of *Leishmania* parasites from patients of the field areas who do not have easily access to health care centers for specialized treatment strategies.

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Introduction

Cutaneous leishmaniasis (CL) is a noticeable tropical disease which has lots of adverse effects on human health in 98 countries on 5 continents in the world as well as Iran.[1,2] The mammalian host(s) of CL in the Old World including Rodentia, Carnivora and human has been infected by different species of single-celled *Leishmania* parasites. Humans are naturally infected by the bite of female sand flies from vertebrate animals in the form of zoonotic cutaneous leishmaniasis (ZCL) with the exception of *Leishmania tropica* which is often known as an anthroponotic transmissible disease from humans to vertebrate animals.[3,4] An epidemiological study of ZCL in 11 provinces of Iran showed that *L. major* was the causative agent of CL in rural areas of Iran with 95% prevalence rate whereas *L. tropica* had a distribution rate of 65% in urban districts.[4] Although, papers have been published on the transmission and epidemiological circulation of *Leishmania* parasites in vectors,[5] reservoir hosts[6,7] and humans from different ZCL foci of Iran,[2,8] there is no considerable and sufficiently investigation on leishmaniasis in humans at the center of Iran (Yazd province). Nevertheless ZCL is considered as an endemic disease in Isfahan province adjacent to Yazd, it has also become endemic in several areas in Yazd province at the center of Iran during the last 10 years.[9,10] Therefore, the constant appearance of infectious agents of *Leishmaniasis* within a human population of this focus highlights the direct significant attention to identifying transmission cycles of *Leishmania* parasites and their clinical manifestations in humans of Yazd district.

Leishmania major is one of the leading-off agents causing rural, zoonotic and vector-borne disease and the digenetic form of *Leishmania* life cycle is completed in different species of wild rodents and phlebotomine sandflies as reservoir hosts and vectors respectively in many geographical locations where it occurs.[10] In fact *L. major* is known as the causative agent of ZCL and more frequent than the other *Leishmania* parasites in Iran which induces Th2 immune response in case of exacerbation with disfigured cutaneous patterns.[11] In addition, another principal agent of cutaneous leishmaniasis, *L. tropica*, has been implicated in occasional cases of recidivans or viscerotropic cases.[12] Recently, *L. tropica* has stimulated many interests because of its potential traits to visceralize and/or classical visceralize in humans,[13] disseminating of cutaneous leishmaniasis along with visceral leishmaniasis[14] and developing of mucosal leishmaniasis in Iran.[15] An epidemiological study of ZCL in 11 provinces of Iran.[15] Accordingly, finding non-native species of *L. tropica* is of great importance in studied region.

However, three species of *Leishmania* parasites have been incriminated as the causative agents of human leishmaniasis in Iran. They are *L. major*, *L. tropica* and *L. infantum*.[16] Also, some other mammals' *Leishmania* such as *L. turanica*, *L. gerbilli* and *L.* close to *gerbilli* have been reported from Iran.[2] and isolated from reservoir hosts and sand flies vectors in different CL foci of Iran.[6,16]

The popular and practical method for identification of *Leishmania* species is Giemsa stained smears prepared from patients' ulcer with the examination of *Leishmania* amastigotes under microscopic observation.[17] In addition, incrimination of *Leishmania* parasites was previously based on clinical symptoms, vector assessments, and pathogenicity in laboratory animals and growth in media.[9,18] In recent years, *Leishmania* species have firmly been identified because of high sensitivity, more rapid determination and characterization after amplifying targeted genes using PCR, RFLP and sequencing.[15,19]

In our investigations, PCR amplification, RFLP digestion and sequencing of ITS-rDNA genotype were employed to identify all *Leishmania* species isolated from the lesion of suspected patients having serous exudate.

The motivation of this work arises from increasing problems of leishmaniasis in the center district of Iran. In fact, this investigation was conducted to monitor the effectiveness of control measures, to accurate incrimination of *Leishmania* parasites at their species level with molecular analyses, to characterize the species-specific parasites isolated from humans residing in the natural field working regions and also to improve our knowledge about all *Leishmania* species those readily maintain their ecological circulation in the endemic focus of Yazd province.

Methods

Origin and sampling of Leishmania parasites from suspected patients

Within the ZCL focus, prepared samples of suspected patients were collected from 16 surveyed villages of Abarkouh city in Yazd province (Fig. 1). Yazd district is geographically located between 31° 07′ 44″ N and 53° 16′ 57″ E of central Iran. The city proper is situated at an altitude of 1510 m (4,954 ft) above sea level (a.s.l.) and all villages of this region were screened and sampled from suspected patients from 2014 to 2016. The smears firstly isolated from patients' lesion, prepared on the slide (slide fixation) and froze within the days of collection. Samples were then transferred on ice to the Pasteur Institute of Iran, Tehran, for microscopic identification and molecular experiments.

The occurrence of leishmaniasis was very low in four consecutive months (May–August). Whereas we found a sharp increasing rate of CL lesions during the 3rd Quarter 2014 (October–December), sampling was carried out at the time of lesions' appearance in Abarkouh district (Fig. 1). The smears were prepared from the active lesions of patients residing in different rural regions of leishmaniasis throughout Abarkouh district. The personal information, lesion duration, type and the number of lesion, ulcer(s)' location, patients' travelling to endemic areas and also drug consumption were recorded for each patient separately. Expanded set of tools (conventional and then molecular methods) were exerted to recognize samples including presumptive CL parasites based on Evans protocol[17] and were then smeared on two microscopic slides, air dried, fixed with methanol and stained by Giemsa. All collected smears were examined under a light microscope (Nikon YS100) with high magnification (1000×) and identified to *Leishmania* infection microscopically (Fig. 2). The sizes of amastigotes and different morphometric shapes of leish-

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